TITLE

METHOD FOR INHIBITING CELL FUNCTIONING FOR USE IN ANTI-INFLAMMATORY AND ANTI-TUMOR THERAPIES

BACKGROUND OF THE INVENTION

Priority Claim: This application is a Continuation of International Application No. PCT/EP00/04388, filed April 28, 2000, which claims the benefit of European Patent Application No. 99201350.8, filed April 28, 1999.

Field of the Invention

[0001] The invention relates to a method for inhibiting cell functioning for use in anti-inflammatory and anti-tumor therapies in the body of a warm-blooded living being. The invention further relates to a drug to be used in the above method, and to the active substance of said drug.

Description of the Related Art

[0002] Inflammations in the body of a warm-blooded living being, in particular a human being, cause many diseases and disorders, and may even turn out to be life-threatening. Therefore, for many decades already it is a major challenge to the clinician to find an effective therapy in treating inflammatory diseases. Various inflammatory diseases, such as rheumatoid arthritis, multiple sclerosis, glomerulonephritis, diabetes and asthma, are the result of unwanted immune responses. As described, for instance, in a recent survey entitled "Manipulation of the Immune Response" ("Immunobiology", 3rd Edition; C.A. Janeway, P. Travers; publ. Current Biology/ Garland/ Churchill Livingstone 1997; Chapter 13), current treatments for immunological disorders are nearly all empirical in

origin using immunosuppressive drugs identified by screening large numbers of natural and synthetic compounds. According to this survey, these drugs may be divided into three categories, viz. (i) drugs of the corticosteroid family, (ii) cytostatic drugs, and (iii) fungal and bacterial derivatives. In this survey it is noted, that these drugs are all very broad in their actions and inhibit protective functions of the immune system as well as harmful ones. In fact, the ideal immunosuppressive agent would be a drug that targets the specific part of the immune response responsible for causing the relevant tissue injury.

[0003] Consequently, according to this survey, antibodies themselves, by virtue of their exquisite specificity, may offer the best possibility for the therapeutic inhibition of specific immune responses. Such immunosuppressive monoclonal antibodies can act by inhibiting target cell functioning. Their promising potential in immunosuppression has already been established. However, as yet these antibodies are not widely and generally used as anti-inflammatory drugs, largely due to the fact that the appropriate targets have not been identified.

BRIEF SUMMARY

[0004] It is the objective of the present invention to provide a method for inhibiting or controlling target cell functioning, for use in anti-inflammatory and anti-tumor therapies in the body of a warm-blooded living being by administration of a drug, having superior therapeutic properties compared to existing anti-inflammatory and anti-tumor drugs. Various requirements should be imposed on a drug to be used in such therapies, for example, non-toxic, no adverse influence on the host resistance, and highly selective to avoid burdening of non-target tissues and organs with drug material.

BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWING

[0005] FIGURE 1 is a diagram related to Example II, the macrophage activity test.

[0006] FIGURE 2 is a diagram related to Example II, the macrophage phagocytosis test.

DETAILED DESCRIPTION OF A PREFERRED EMBODIMENT OF THE INVENTION

[0007] According to the present invention the above-defined objective can be achieved by a method which comprises administering to said being a drug comprising, in a quantity effective for said therapies, a substance that specifically recognizes the extracellular domain of SIRP (= signal regulatory protein) (anti-SIRP substance) and that inhibits the functioning of pathologic myeloid cells. By using a drug according to the method of the present invention, both a highly selective and an effective therapy in treating inflammatory diseases, in particular autoimmune diseases and allergies, and tumors can be achieved.

[0008] Well-known examples of myeloid cells are macrophages, which are continuously replinished from a population of dividing and maturing myeloid precursor cells in the bone marrow. This ensures the continuous availability of macrophages in all tissues of the body and allows a fast and efficient response in case of infections. In a number of circumstances, however, macrophages do not play a benificial role, which may lead to pathologies. In a variety of autoimmune diseases, like rheumatoid arthritis, multiple sclerosis, glomerulonephritis etc., and allergies, like asthma, activated macrophages play an important role in the induction and/or maintenance of inflammations that, as a consequence, forms the basis for the (general chronical) clinical symptoms. In addition,

under certain circumstances the myeloid precursor cells may cause pathologies; the unlimited growth of these myeloid precursor cells is the cause of certain malignant tumors, in particular myeloid leukemia.

[0009] International patent application publ. no. WO 97/48723 relates inter alia to a method of treating an organism having a disease or condition characterized by an abnormality in a signal transduction (s.t.) pathway, wherein said s.t. pathway includes an interaction between inter alia a SIRP polypeptide and a natural binding partner, comprising the step of promoting or disrupting said interaction (claim 25). This is explained in greater detail in page 37, lines 5-27 of WO 97/48723. There is no indication, that the inhibition the of functioning of pathologic myeloid cells according to the method of the invention underlying the present application is causally connected with any interaction between a SIRP substance and a natural binding partner and any influence (promotion or disruption) of such an interaction.

[0010] More in particular, said anti-SIRP substance to be used in the method of the present invention is characterized in that it inhibits the functioning of macrophages by suppressing their activation by a factor of at least 10 as measured by each of the following so-called macrophage activity tests: (i) the production of nitric oxide (NO), (ii) the production of reactive oxygen species, in particular superoxides (e.g. H_2O_2), and (iii) the production of tumor necrosis factor - alpha (TNF). The above tests for measuring the activity of macrophages are described in detail in Example II hereinafter. It has been found, that the substances to be used according to the method of the invention show the above striking effect in all three above tests. Therefore these tests are a convenient tool of distinguishing substances within the scope of the invention from other compounds.

[0011] Signal-regulatory proteins (SIRP), as recently disclosed by S. Adams et al. in J. Immunol. 161: 1853-1859 (1998), are transmembrane glycoproteins, inhibiting signaling through receptor tyrosine kinases but having a physiological function which is unknown up to the present. SIRP is selectively expressed on the surface of myeloid cells, such as macrophages, monocytes, granulocytes and dendritic cells, and neurons. The active substances to be used in the method of the present invention can be any compound, e.g. proteinaceous substances having a polypeptide main-chain or low molecular weight substance. According to the present invention these substances should meet the following requirements:

(1) specific recognition of (the extracellular domain of) SIRP, according to a test method as disclosed in the above publication by Adams et al.; and

(2) suppression of the activation of macrophages according to the three tests as described above.

[0012] As mentioned above, the functioning of myeloid precursor cells may cause pathologies; an unlimited growth or division of myeloid precursor cells is the cause of myeloid leukemia, a malignant tumor. It has been found that the above anti-SIRP substances to be used according to the method of the invention can also inhibit the functioning of pathologic myeloid cells by strongly suppressing the division of macrophage tumor cell lines. More specifically, the suppression of this cell division is also found to amount to a factor of at least 10, as demonstrated in the so-called macrophage division test (Examples II). Therefore, a drug comprising said anti-SIRP substance in an effective quantity can successfully be used in anti-tumor therapy, in particular for treating myeloid leukemia, because the selective binding of these

substances to the extracellular domain of SIRP can effectively and selectively control the division of myeloid cells.

[0013] The above-mentioned functioning of myeloid cells, in particular macrophages, encompasses not only their activation and division, but also the phenomenon of phagocytosis, that is the uptake of other organisms or other particles. In case of gene-targeted therapies, e.g. gene-targeted anti-tumor therapy, where genes packed in vector particles (vehicles) are targeted to different cells or tissues, macrophages with their potent phagocytic capacity are a major barrier in the efficient delivery of the genes of interest. The method of the present invention is to be considered to also encompass a method for use in such gene-targeted therapies. If these therapies are attended by a drug comprising the above anti-SIRP substance, a common pathway of macrophage phagocytosis can be inhibited, resulting in a temporal suppression of said phagocytosis and consequently in a considerable improvement of the efficacy of these therapies. This unique property in gene-targeted therapies makes the active substances according to the present invention extremely useful therapeutically: see the results of the so-called macrophage phagocytosis test in Example II.

[0014] More specifically, the anti-SIRP substances to be used according to the method of the present invention, can be characterized in a preferred embodiment as being selected from the group consisting of Fab-fragments of monoclonal antibodies and (bio)chemically modified products of such fragments wherein the intended anti-SIRP activity has been maintained. Suitable examples of such modified products of said Fab-fragments are NH-acylated products, S-S - reduced products comprising free mercapto groups, etc., provided that the intended activity has been maintained.

[0015] It has been found, that Fab-fragments of the monoclonal antibodies ED9 and ED17, as well as the above-mentioned modified products thereof, are extremely promising for the therapeutic method of the invention, as can be concluded from a number of suitable cell culture experiments that are predictive for human application. These experiments are described in the accompanying Examples. From the results of these experiments it will be evident, that the tested anti-SIRP substances have properties, which make them particularly suitable for use in the method of the invention.

[0016] The above monoclonal antibodies ED9 and ED17 are described in the above-mentioned publication by Adams et al., as well as their selective recognition of rat SIRP (anti-SIRP activity) that is selectively expressed by myeloid cells, e.g. by macrophages. These authors have found, that the binding of these monoclonal antibodies to macrophages induces the production of nitric oxide (NO). It is indeed quite a surprise, that the inventors of the present invention have found, that the Fab-fragments of the same monoclonal antibodies ED9 and ED17 show an opposite effect after binding to macrophages, viz. a suppression of the production of nitric oxide. It is precisely this effect that makes the anti-SIRP substances of the present invention so suitable for the intended use.

[0017] The present invention also relates to a drug for inhibiting cell functioning for use in anti-inflammatory and anti-tumor therapies, as indicated above. Such a drug according to the present invention comprises, in addition to a pharmaceutically acceptable carrier and, if desired, one or more pharmaceutically acceptabe adjuvants, as the active ingredient an anti-SIRP substance that inhibits the functioning of pathologic myeloid

cells. The above-mentioned solid or liquid carriers, as well as the suitable adjuvants are well-known in pharmacy.

[0018] In a preferred embodiment said drug according to the present invention comprises an anti-SIRP substance selected from the group consisting of Fab-fragments of monoclonal antibodies, preferably of ED9 or ED17, and (bio)chemically modified products of such fragments wherein the intended anti-SIRP activity has been maintained.

[0019] Furthermore the present invention relates to an anti-SIRP substance that inhibits the functioning of pathologic myeloid cells, said anti-SIRP substance being selected from the group consisting of Fab-fragments of monoclonal antibodies, preferably of ED9 or ED17, and (bio)chemically modified products of such fragments wherein the intended anti-SIRP activity has been maintained.

[0020] Finally the present invention also relates to a method to detect a substance interacting with SIRP and inhibiting the functioning of pathologic myeloid cells, said method comprising the steps of:

1. providing a cell line expressing SIRP on its membrane;
2. stimulating the production of pro-inflammatory cytokines;
3. contacting the substance of interest with the stimulated cell line, and
4. measuring the change in production of inflammatory mediators.

[0021] The test cell-line preferably is of human origin. SIRP may be naturally expressed in the cell line. However, expression might also be accomplished by insertion of the gene encoding SIRP or part thereof such as the extracellular domain. In the present method cell lines expressing chimeric proteins (e.g. rat-human, mouse-human chimeras) might be used as well. Under inflammatory mediators is to be understood H_2O_2, N0, pro-

inflammatory cytokines such as e.g. TNF-a, IL-6 or IL-8 or pathway intermediates resulting in expression and/or secretion of the pro-inflammatory cytokines. The cell lines to be used in the screenings method can be stimulated to produce pro-inflammatory cytokines by macrophage activating molecules such as LFS or IFN-g.

[0022] The invention will now be described in greater detail with reference to the following specific Examples.

Example I

[0023] Preparation of Fab-fragments of ED9 and ED17.

[0024] The starting monoclonal antibodies ED9 and ED17 are disclosed by Damoiseaux et al. in J. Leukocyte Biol. 46:556-564 (1989) and 49: 434-441 (1991). The Fab-fragments of these antibodies are obtained by papain-protolytic digestion. For this purpose a papain-solution, containing 0.1 mg of papain per ml PBS buffer solution (0.02M EDTA and 0.02M cystein in PBS), is added to the same volume of a solution of the antibody (1 mg/ml) in PBS. The mixture is incubated at 37°C, and after a certain time, determined by making a time-series, the reaction is stopped by adding a 0.03M iodoacetamide solution (addition of 20 μl 0.3M iodoacetamide to 110 μl incubated mixture). The mixture is now dialysed against 2 1 PBS at pH 8.0, 0/N at 4°C. The solution is chromatographed over a protein A sepharose column, concentrated to 5 ml at reduced pressure, and chromatographed over a superose 12 column. The fractions of 50 kD are received and purity-controlled on non-reduced SDS-PAGE^{R}. The solution of the Fab-fragments ED9 and ED17, so obtained, are used as such in the cell culture experiments described in Example II.

Example II

Cell culture experiments

The macrophage activity test

[0025] Rat peritoneal macrophages, obtained by peritoneal lavage, of the rat macrophage cell line NR8383 (Adams et al. 1998) are cultured at a density of 0.25 x 10^6 cells/ml in RPMI-1640 medium containing 2% fetal calf serum and 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. Macrophage activating stimuli (100 ng/ml lipopolysaccaride (LPS), or 20 U/ml gamma-interferon (IFN)-g) are added in the presence (or absence) of anti-SIRP Fab-fragments (ED9 or ED17; 40 μg/ml) or control Fab-fragments (OX41, Adams et al. 1998; 40 μg/ml). After 18-20 hours the cell culture supernatants (separated from the cells by centrifugation for 7 min. at 500g) are harvested. NO production in supernatants is measured using Griess reagent (Ding et al. (1988), J. Immunol. 141:2407) using NaNO2 to produce a calibration curve. TNFα, IL1ß and IL6 are measured by enzyme-linked immunosorbent assay as described (Vincent et al. (1996), Glia 17:94; Lenczowski et al. (1997), Am.J.Physiol. 273:R1870). The results are presented in the diagram of Figure 1.

The macrophage phagocytosis test

[0026] 0.5 x 10^6 rat peritoneal macrophages are plated in each well of a 24-well cell culture plate in RPMI-1640 medium containing 10% fetal calf serum and 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin, and are then allowed to adhere for 1-1.5 hours at 37°C in a 5% CO_2 atmosphere. After this the cells are washed 2x and incubated with 0.5 ml HEPES (25 mM)-buffered RPMI containing 2 μg oxidated LDL (low-density lypoproteins), 2 μg acetylated LDL (both: FITC-labelled; Molecular

Probes), 1 μl latex beads (FITC-labelled; Molecular Probes), 2 μg serum treated zymosan (FITC-labelled), or rat myelin (DiI-labelled) plus 5% fresh rat serum. These incubations are performed in the presence (or absence) of anti-SIRP Fab-fragments (ED9 or ED17; 40 μg/ml) or control Fab-fragments (OX41; 40 μg/ml). After 1.5 hours the cells are washed to remove non-bound particles, cells are detached by incubation in 5 mM EDTA in PBS and mean fluorescence intensity for each cell is measured on a FACScan^R. Values are plotted as the percentage of control phagocytosis: Figure 2. FITC and DiI are fluorescent dyes, well-known in the art.

The macrophage division test

[0027] The rat macrophage cell line NR8383 (Adams et al. 1998) are cultured at a density of 0.25 x 10^6 cells/ml in a 96-well cell culture plate in RPMI-1640 medium containing 2% fetal calf serum and 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. This is performed in the presence (or absence) of anti-SIRP Fab-fragments (ED9 or ED17; 40 μg/ml) or control Fab-fragments (OX41; 40 μg/ml). After 24 h ^{3}H-thymidine (1 μCi/well) is added and the cells are incubated for another 6 hours. The cells are harvested using a cell harvester and cell incorporated radioactivity is determined in a Micro-ß-plate reader. The mean results are shown in Table 1 below:

Treatment	Mean (in c.p.m.)	SD (standard dev.)
control	132783	2730
ED17 Fab	6845	197
OX41 Fab	154889	8528

Results

[0028] In all above experiments the results of ED9 Fab and of ED17 Fab are comparable with each other; therefore the results presented are confined to one active substance. To evaluate the effects of ED9 or ED17 Fab-fragments, cell culture experiments using animal cells, predictive for human myeloid and/or inflammatory cells, are performed. In the macrophage activity test (Figure 1) the effect on the production of the inflammatory mediators reactive oxygen species (H_2O_2 as ROS), nitric oxide (NO) and the proinflammatory cytokine TNFα is measured. As can be seen, ED9 Fab strongly suppresses the production of ROS (not shown), NO and TNFα, whereas irrelevant OX41 Fab-fragments do not have this effect. To evaluate the effect of ED9 or ED17 Fab on phagocytosis peritoneal macrophages are assayed as described in the macrophage phagocytosis test. As can be seen (Figure 2), ED17 Fab strongly suppresses the phagocytosis of various particles, including myelin+serum, serum-treated zymosan, latex beads, and oxydated- or acetylated-low density lipoproteins. Again control OX41 Fab fragments had no such effects.

[0029] To examine the effects of ED9 or ED17 Fab on myeloid cell division, NR8383 cells are assayed as described in the macrophage division test. As illustrated in table 1, ED strongly inhibits division (analyzed by thymidine incorporation), whereas OX41 Fab has little effect.

Discussion and conclusion

[0030] Taken together, these results show that Fab fragments of antibodies ED9 or ED17, directed against an overlapping epitope of SIRPα (Adams et al. 1998), can potently suppress the activation and phagocytosis of macrophages and the cell division of myeloid

cells. No such effects are seen with Fab fragments of other antibodies, directed against a different SIRPα epitope (OX41). These properties make the extracellular domain of SIRPα a target for: (1) anti-inflammatory therapy and (2) anti-tumor therapy of myeloid leukemia. In addition, (3) temporal suppression of phagocytosis via SIRPα ligation can help to increase the efficiency of gene therapy.

[0031] Figure 1. Anti-SIRPα Fab fragments (ED9) inhibit the production of (a) NO and (b) TNFα induced by LPS (100 ng/ml) or IFN-γ (20 U/ml) in NR8383 macrophages.

[0032] Figure 2. Anti-SIRPα Fab fragments (ED17) inhibit the phagocytosis by peritoneal macrophages of various particles, including myelin+serum, serum-treated zymosan, latex beads, and oxydated- or acetylated-low density lipoproteins.

[0033] Table 1. Anti-SIRPα Fab fragments (ED17) inhibit the division of myeloid NR8383 cells.